Supporting Information

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SI Experimental Procedures

Materials and Cell Lines. Lenalidomide (Chem-Pacific), suberoylanilide hydroxamic acid (SAHA/Vorinostat; Sigma), MLN4924 (Pevonedistat; Active Biochem), MG132 (Millipore), NMS-873 (Calbiotech), and CB-5083 (a kind gift from Seth Cohen, University of California, San Diego, La Jolla, CA) were dissolved in DMSO at room temperature and were stored at −80 °C until use. MSO and nicotinamide (NAM) from Sigma were dissolved in distilled water and kept at −80 °C and 4 °C, respectively.

NCI-H1299 cells (ATCC no. CRL-5803), MCF7 cells (ATCC no. HTB-22), MDA-MB-231 cells (ATCC no. HTB-26), and HEK293 cells (CRL-1573) were purchased from ATCC. *CRBN*-knockout 293FT cells were kindly provided by William Kaelin, Dana Farber Cancer Institute, Boston. Cells were grown in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS (Atlanta Biologicals), 2 mM glutamine, and penicillin-streptomycin. Human multiple myeloma cell lines, including MM.1S and U266 purchased from ATCC and L363 cells (kindly provided by Francesco Parlati, Calithera Biosciences, South San Francisco, CA) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% FBS supplemented with 2 mM glutamine and penicillin-streptomycin. All cell cultures were checked periodically for mycoplasma contamination. Cell lines also were authenticated by short tandem repeat DNA profiling analysis by Laragen, Inc.

Antibodies. Anti-glutamine synthetase (C-20; sc-6640-R), anti-GAPDH conjugated to HRP (FL-335 HRP; sc-25778 HRP), anti-IKZF1 (H-100; sc-13039), and anti-VCP/p97 (H-120; sc-20799) antibodies used for Western blots were from Santa Cruz Biotechnology. Anti-NPL4 (13489) and Anti-UFD1 antibodies (13789) were from Cell Signaling Technology. Anti-ubiquitin (P4D1-A11; 05–944) and anti-UBXD7 (AB10037) antibodies were from EMD Millipore. Anti-CRBN (HPA045910) and anti-Myc HRP (A5598) antibodies were from Sigma. Anti-IKZF3 antibody (NBP2-24495) was from Novus Biologicals. Anti-Flag HRP-conjugated antibody (600-403-383) was from Rockland Immunochemicals. For secondary antibodies, HRP goat anti-rabbit IgG (PI-1000) and HRP horse anti-mouse IgG (PI-2000) were from Vector Laboratories. For IP experiments, anti-VCP antibody (ab11433) was from Abcam, and EZview Red anti-Flag M2 (F2426) and EZview Red anti–c-Myc affinity gels (E6654) were from Sigma.

RNAi-Mediated Knockdown. For siRNA transfection, control (nontargeting; sc-78528), NPL4 (sc-61227), UFD1 (sc-41689), and UBXD7 (sc-78377) siRNAs were purchased from Santa Cruz Biotechnology. Cells were grown to 50% confluence in 10-cm plates and then were transfected with 50 nM (final concentration) of siRNAs using Opti-MEM medium and Lipofectamine RNAiMAX transfection reagent according to the manufacturer's protocol (Thermo Fisher Scientific). To achieve maximally effective siRNA knockdown, cells were transfected again 24 h later. Beginning 24 h after the second siRNA transfection, cells were starved of glutamine for 24 h, followed by the addition of 4 mM glutamine. Subsequently, cells were harvested and analyzed. For shRNA-mediated knockdown of p97, the HEK-293-p97sh cell line (DTC-139) expressing doxycycline-inducible p97-specific shRNA from the pTRIPZ-p97sh lentiviral construct was used as previously described (73).

Plasmids. Lentiviral vectors directing the expression of WT ^{Flag}CRBN (RDB2915: pCDH-Flag-CRBN) and its mutants (RDB3186: pCDH-Flag-CRBN-W400E; RDB3187: pCDH-Flag-CRBN-N351R), constructed in pCDH-T2AcGFP-MSCV (System Biosciences), were recently reported (24). pcDNA3.1-^{3xFlag}NPL4 plasmid (RDB3314) and pcDNA3.1-UFD1^{1xFlag} plasmid (RDB3315) were a kind gift from David Chan (California Institute of Technology, Pasadena, CA) (74). pcDNA3.1-p97$^{Myc-His}$ WT (RDB2001) and pcDNA3.1-p97$^{Myc-His}$ E578Q mutant (RDB2002) plasmids were a gift from Phyllis Hanson, Washington University in St. Louis, St. Louis (75). The full-length cDNA of IKZF1-V2 was amplified from the lentiviral vector plenti-UBCgate-IKZF1-V2-3xHA-pGK-PUR (a kind gift from William Kaelin) (29) and then was subcloned into pCMV6-Flag-Myc tags (pCMV6-IKZF1$^{Flag-Myc}$; RDB3318). All cDNAs cloned into mammalian expression vectors were confirmed by DNA sequencing (Laragen).

Immunoblot Analysis and IP. The protocols were performed as described previously (24, 76).

For Fig. 6*E*, U937 (ATCC) and MM1S (ATCC) cells were maintained in RPMI 1640 tissue-culture medium (Invitrogen) supplemented with 10% FBS, 1× sodium pyruvate, 1× nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubation with DMSO, CB5083, MLN4924, or MG132 for 1 h, cells were treated with CC-885 for an additional 2 h. Cells then were washed twice with ice-cold 1× PBS and were lysed in buffer A [50 mM Tris·HCl, 150 mM NaCl, 1% Triton X-100, a Complete Protease Inhibitor tablet (Roche), and a Phosphatase Inhibitor Cocktail tablet (Roche)]. Whole-cell extracts were harvested and subjected to immunoblot analysis with the following antibodies: rabbit anti-GSPT1 polyclonal antibody (ab49878; Abcam), rabbit anti-CRBN monoclonal antibody (CRBN65; Celgene), mouse anti-Actin monoclonal antibody (A5316; Sigma-Aldrich), goat anti-mouse IRDye-800 antibody (926–32210; LI-COR), and goat anti-rabbit IRDye-800 antibody (926–32211; LI-COR).

Cycloheximide Chase Experiments. MM.1S cells were seeded overnight in complete medium in 24-well plates (1×10^5 cells per well) and then were pretreated with CB-5083 (2 μM) and/or lenalidomide (10 μM) for 30 min, followed by the addition of 100 μg/mL cycloheximide. At the indicated times after the addition of cycloheximide, samples were harvested for immunoblot analysis.

TUBE2 Pulldown. Cells grown to 80% confluence in 15-cm plates and starved of glutamine for 24 h were pretreated with the proteasome inhibitor MG132 (10 μM), the NEDD8-activating enzyme inhibitor MLN4924 (1–2 μM), or the p97 inhibitors CB-5038 (10 μM) or NMS-873 (10 μM) for 0.5 h, followed by the addition (or not) of 4 mM glutamine for 2–4 h. The cells were lysed in IP lysis buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100] containing a protease inhibitor mixture, MG132 (10 μM), and 10 mM *N*-ethylmaleimide (NEM; Sigma). Whole-cell protein extracts were incubated with 20 μL of TUBE2 agarose beads (Boston Biochem) for 2 h with rotation at 4 °C. Beads were washed three times with IP lysis buffer, and bound proteins were eluted in 60 μL 1.5× SDS sample buffer, boiled for 5 min, and subjected to Western blot analysis.

For USP2 treatment, after washing five times with IP lysis buffer and two times with ubiquitylation buffer [50 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 1 mM DTT] plus the protease inhibitor mixture MG132 (20 μM), the beads were incubated at

30 °C for 2 h in 30 μL of ubiquitylation buffer containing 1.5 μL USP2 (E-504; Boston Biochem). USP2-treated samples were mixed with 30 μL 2× SDS sample buffer, boiled for 5 min, and subjected to Western blot analysis.

In Vivo Ubiquitylation Assays. The in vivo ubiquitylation assays were performed under denaturing IP conditions (24, 76). HEK293 cells were grown in 10-cm plates to 60% confluence and then were transiently transfected with 4 μg pCMV6-IKZF1$^{Flag\text{-}Myc}$ and 0.5 μg pcDNA3- ^{HA}Ubiquitin (^{HA}Ub) and/or empty vector plasmid (pcDNA3.1) using FuGENE-HD transfection reagent (Promega). After 24 h of transfection, the cells were pretreated with MG132 (20 μM) or CB-5083 (10 μM) for 30 min followed by the addition of 10 μM lenalidomide for 3 h. Cells were harvested using trypsin, lysed in 0.3 mL denaturing IP lysis buffer [1% SDS, 50 mM Tris, 10 mM DTT (pH 7.5)], and boiled for 5 min. Subsequently, denatured proteins were diluted 10× in native IP lysis buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100] containing a protease inhibitor mixture, MG132 (10 μM), and 10 mM *N*-ethylmaleimide (NEM) (Sigma) and then were immunoprecipitated with anti-Flag resin. IP washing steps were performed using IP lysis buffer supplemented with 0.3 M NaCl. The input lysates and bound fractions were evaluated by SDS/PAGE and immunoblotting with antibodies against the HA and Flag tags.

Gel Filtration. Cells grown in 15-cm plates (~50–60% confluence) were starved of glutamine by culturing in glutamine-free DMEM supplemented with 10% FBS and penicillin/streptomycin for 24 h and then were pretreated with different inhibitors, including SAHA+NAM, MLN4924, MSO, MG132, NMS-873, and CB-5083, for 30 min, followed by the addition of 4 mM glutamine for 2 h (short treatment) or 6 h (long treatment). Cells were harvested and lysed in 1 mL of cell lysis buffer [25 mM Tris (pH 7.5), 250–300 mM NaCl, 1% Triton X-100] containing a protease inhibitor mixture, 1 mM DTT, and 10 mM NEM. Cell lysates were sonicated briefly and cleared by centrifugation at 18,000 × *g* for 10 min at 4 °C. After filtering through a 0.22-μm Ultrafree-MC centrifugal filter device (Millipore), soluble protein extracts (500 μL) were applied to a Superdex 200 10/300 GL (GE Healthcare Life Sciences) gel filtration column connected to an ÄKTA FPLC system (GE Healthcare Life Sciences). The void volume was determined using blue dextran (Sigma; D5751) as shown in Fig. S7*A*. MW estimation is based on the protein standard profile generated in house, as shown in Fig. S7*B*. Individual fractions of 1 mL in protein storage buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT] were collected and concentrated by 10% TCA precipitation, followed by washing with 1 mL ice-cold acetone. The pellets were air-dried and resuspended in 100 μL 1.5× SDS sample buffer, boiled for 5 min, and subjected to Western blotting analysis with the indicated antibodies.

Assessment of *CRBN* and p97 Complex Covariance. RNA-sequencing (RNA-seq) transcripts per million (TPM) values were obtained for CCLE cell lines. These data were generated by the Cancer Target Discovery and Development (CTD^2) network (https://ocg.cancer.gov/programs/ctd2/data-portal) established by the National Cancer Institute's Office of Cancer Genomics (https://ocg.cancer.gov/ctd2-data-project/translational-genomics-research-institute-quantified-cancer-cell-line-encyclopedia). Original data and scripts used to retrieve data are available upon request. The significance of Crbn covariance (*P* value) with all detected transcripts was calculated from Pearson's correlation coefficients. To determine if the group of p97 and its adaptors covary significantly with Crbn, Kolmogorov–Smirnov testing ($\alpha = 0.01$) was used. The distribution of all transcripts' covariance with *CRBN* was determined to be significantly different from the distribution of *CRBN* covariance with p97 and its adaptors, indicating that p97 and its adaptors covary significantly with Crbn expression.

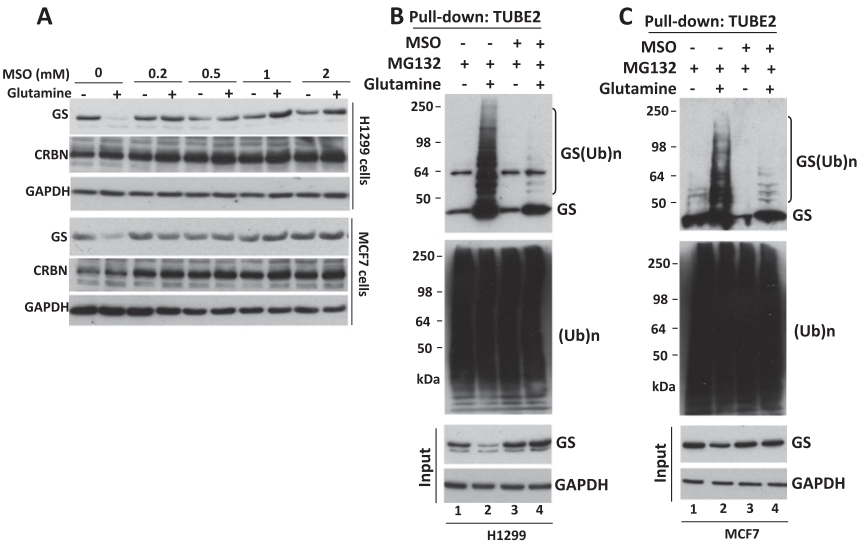

Fig. S1. (Related to Fig. 1) The GS inhibitor MSO suppresses glutamine-induced GS ubiquitylation and degradation. (*A*) H1299 and MCF7 cells were starved of glutamine for 24 h. Cells were pretreated with the GS inhibitor MSO for 30 min, followed by the addition (or not) of 4 mM glutamine for 8 h. Cell extracts were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, CRBN, and GAPDH. (*B* and *C*) H1299 (*B*) and MCF7 (*C*) cells were starved of glutamine for 24 h and then were pretreated with MG132 (10 μM) in the presence or absence of MSO (1 mM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 3 h. Cell lysates were fractionated on a TUBE2 resin. The bound fractions and lysate samples (inputs) were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin, and GAPDH. (Ub)n, polyubiquitin.

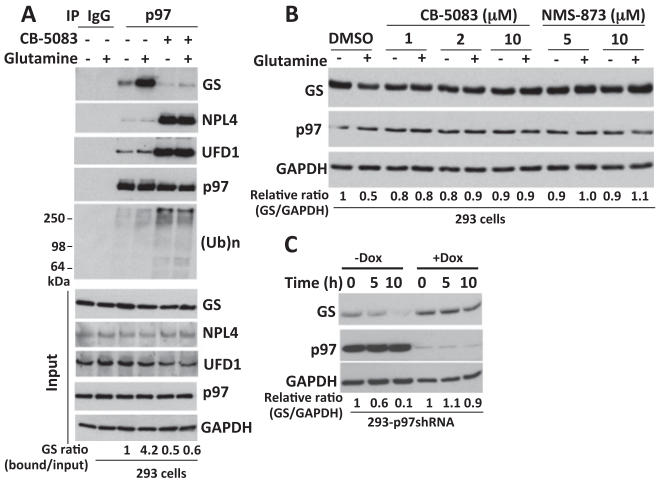

Fig. S2. (Related to Fig. 2) p97 interacts with endogenous GS and is required for glutamine-induced GS degradation. (*A*) HEK293 cells were starved of glutamine for 24 h and were pretreated (or not) with CB-5083 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. Protein extracts were immunoprecipitated with mouse IgG control or p97 antibodies, followed by Western blot analysis with the indicated antibodies. The ratios of GS bound to p97 normalized to input GS are shown. (Ub)n, polyubiquitin. (*B*) HEK293 cells were starved of glutamine for 24 h and were pretreated with the p97 inhibitor NMS-873 or CB-5083 for 30 min, followed by the addition (or not) of 4 mM glutamine for 4 h. Cell extracts were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, p97, and GAPDH. The relative ratio of GS:GAPDH, normalized to lane 1, is shown. (*C*) HEK293 cells stably expressing doxycycline (Dox)-inducible shRNA targeting p97 were mock-treated or were induced with doxycycline (1 μg/mL) for 48 h and then were starved of glutamine for 24 h, followed by the addition of 4 mM glutamine and the HDAC inhibitors (2 μM SAHA+10 mM NAM) for the indicated times. Cell extracts were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, p97, and GAPDH. The GS:GAPDH ratio for each sample was calculated, normalized to untreated cells, and is indicated below the bottom immunoblot in each panel.

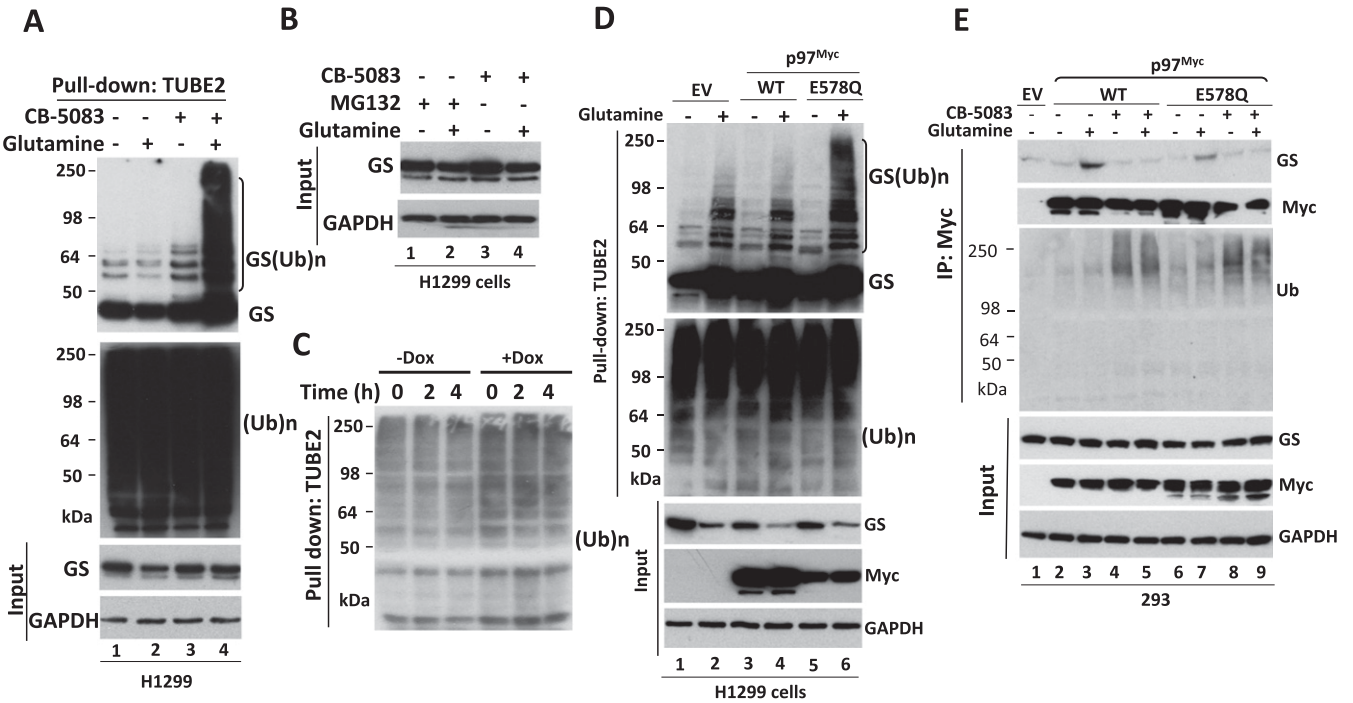

Fig. S3. (Related to Fig. 2) p97 controls GS ubiquitylation status in response to glutamine. (*A*) H1299 cells were starved of glutamine for 24 h and then were pretreated with CB-5083 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 3 h. Cell lysates were fractionated on a TUBE2 resin. The bound fractions and lysate samples were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin, and GAPDH. (Ub)n, polyubiquitin. (*B*) Western blot analysis showing input protein levels for Fig. 3*B*. (*C*) The bound fractions shown in Fig. 2*F* were analyzed by SDS/PAGE and immunoblotting with anti-ubiquitin antibody. (*D*) The ATP hydrolysis-deficient p97 E578Q mutant promotes the accumulation of ubiquitylated GS in response to glutamine. H1299 cells were transfected with empty plasmid (EV) or with plasmids expressing WT $p97^{Myc}$ or the $p97^{Myc}$-E578Q mutant. Twenty-four to thirty-six hours after transfection, cells were starved of glutamine for 24 h, followed by the addition (or not) of 4 mM glutamine for 2 h. Total ubiquitinated proteins were affinity-purified using TUBE2-agarose. Bound fractions and cell lysates (input) were analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. (*E*) The ATP hydrolysis-deficient p97 E578Q mutant binds endogenous GS. HEK293 cells were transfected with empty plasmid or with plasmids expressing WT $p97^{Myc}$ or the $p97^{Myc}$-E578Q mutant. Twenty-four hours after transfection, cells were starved of glutamine for 24 h and were pretreated (or not) with CB-5083 (10 μM) for 30 min followed by the addition (or not) of 4 mM glutamine for 2 h. Cell extracts were immunoprecipitated with anti-Myc antibody, and the precipitated and input fractions were evaluated by SDS/PAGE and immunoblotting with the indicated antibodies.

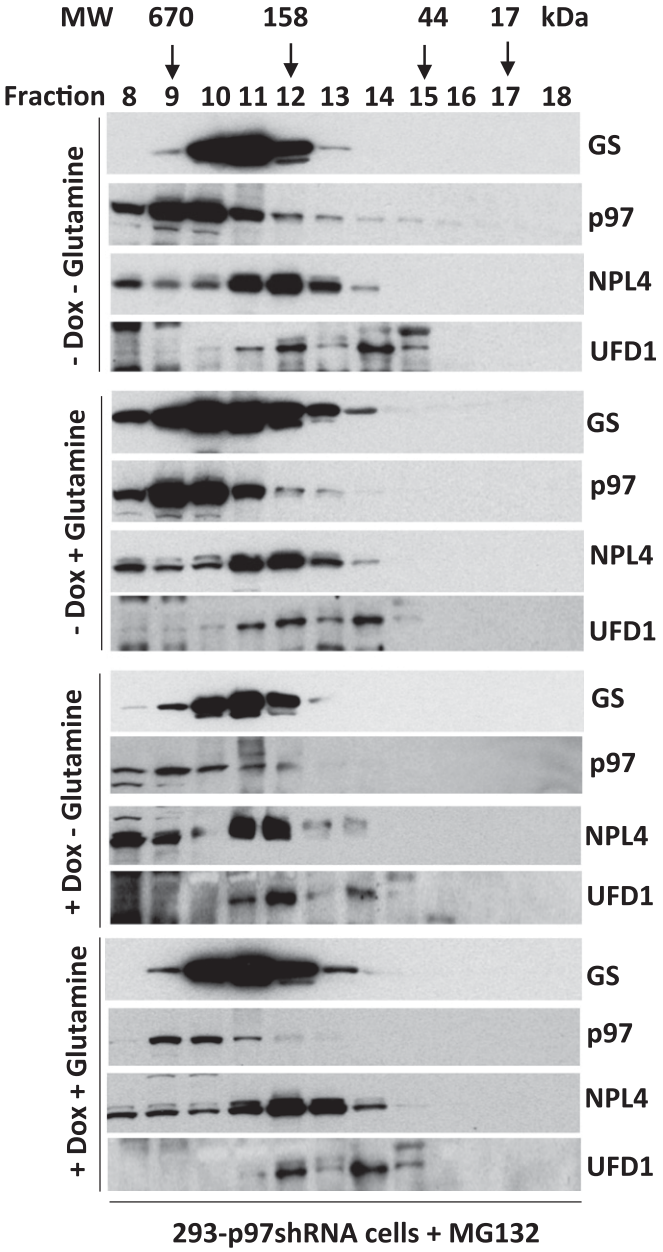

Fig. S4. (Related to Fig. 5) Depletion of p97 by shRNA blocks glutamine-induced GS disassembly. HEK293 cells stably expressing doxycycline (Dox)-inducible shRNA targeting p97 were mock-treated or were induced with doxycycline (1 μg/mL) for 48 h, starved of glutamine for 24 h, and then pretreated with MG132 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 6 h. Cell lysates were fractionated on a Superdex 200 gel filtration column. Individual fractions were concentrated by TCA precipitation and analyzed by SDS/PAGE and immunoblotting with the indicated antibodies.

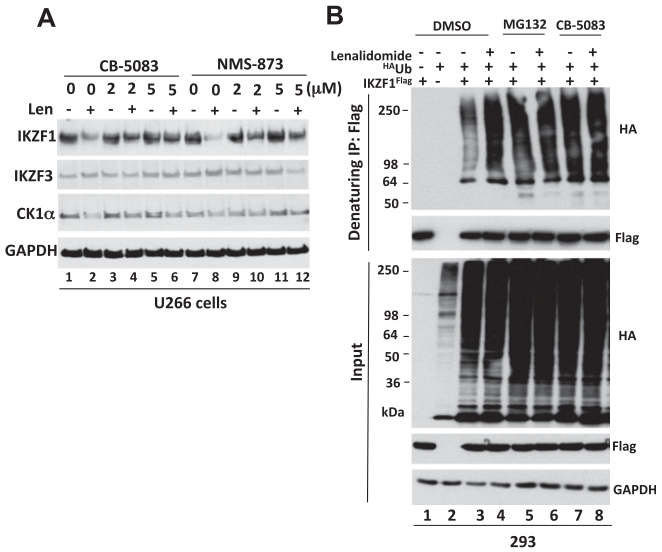

Fig. S5. (Related to Fig. 6) (*A*) Lenalidomide-induced degradation of CRBN neosubstrates requires p97. U266 cells were pretreated with the indicated doses of CB-5083 or NMS-873 for 30 min, followed by the addition (or not) of lenalidomide (Len) (10 μM) for 4 h. Cell lysates were fractionated by SDS/PAGE and immunoblotted for the indicated endogenous proteins. (*B*) Depletion of p97 or proteasome activity promotes the accumulation of ubiquitylated IKZF1, suggesting that p97 functions downstream of IKZF1 ubiquitylation. HEK293 cells were transfected with plasmids encoding ^{HA}Ub and IKZF1^{Flag}. After 24 h of transfection, the cells were pretreated with 20 μM MG132 or 10 μM CB-5083 for 30 min followed by the addition (or not) of 10 μM lenalidomide for 3 h. Denatured lysate proteins were immunoprecipitated with anti-Flag antibody. The input lysates and bound fractions were evaluated by SDS/PAGE and immunoblotting with antibodies against the HA and Flag tags.

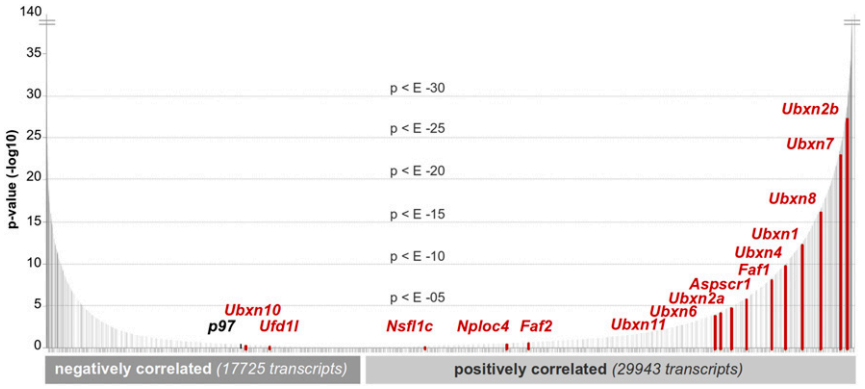

Fig. S6. (Related to Fig. 3) *CRBN* expression covaries significantly with p97 and its adaptors. Transcript expression values (expressed as TPM) were obtained from RNA-seq performed on cancer cell lines in the 935 CCLE. These data were generated by the CTD^2 network (https://ctd2.nci.nih.gov/dataPortal/) established by the National Cancer Institute's Office of Cancer Genomics. For each detected transcript, significance of correlation with Crbn across 935 samples was determined. Data are plotted in terms of increasing significance for transcripts positively and negatively correlated with *CRBN*. p97 and its adaptors, as a whole, are significantly correlated with *CRBN* mRNA expression as determined by Kolmogorov–Smirnov testing.

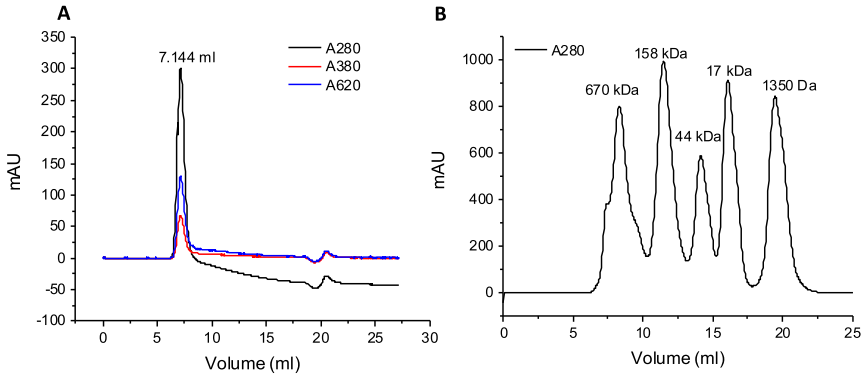

Fig. S7. (*A*) Void volume determination of Superdex 200 10/300 GL. Blue dextran (400 μL, 1 mg/mL) was injected into a Superdex 200 10/300 GL column and was monitored at wavelengths of 280, 380, and 620 nm (A280, absorbance at 280 nm; A380, absorbance at 380 nm; A620, absorbance at 620 nm). (*B*) Chromatogram for protein standard of Superdex 200 10/300 GL. Gel filtration standard (500 μL) (Bio-Rad; 151–1901) was injected into a Superdex 200 10/300 GL column and was monitored at a wavelength of 280 nm. mAU, milli absorbance unit.